Catalytic antibody degradation of ghrelin increases whole-body metabolic rate and reduces refeeding in fasting mice

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Obesity is a chronic, costly, and globally prevalent condition, with excess caloric intake a suspected etiologic factor. Nonsurgical treatments are modestly efficacious, and weight loss maintenance is hampered by anti-famine homeostatic mechanisms. Ghrelin, a gastric hormone linked to meal initiation, energy expenditure, and fuel partitioning, is hypothesized to facilitate weight gain and impede weight loss. Unique among known animal peptides, the serine-3 residue of ghrelin is posttranslationally acylated with an *n*-octanoic acid, a modification important for the peptide's active blood-brain transport and growth hormone secretagogue receptor-1 agonist activity. Pharmacological degradation of ghrelin would be hypothesized to reduce ghrelin's biological effects. To study endogenous ghrelin's role in appetite and energy expenditure, we generated antibodies that hydrolyze the octanoyl moiety of ghrelin to form *des*-acyl ghrelin. The most proficient antibody catalyst, GHR-11E11, was found to display a second-order rate constant of 18 $M^{-1}{\cdot}s^{-1}$ for the hydrolysis of ghrelin to *des*-acyl ghrelin. I.v. administration of GHR-11E11 (50 mg/kg) maintained a greater metabolic rate in fasting C57BL/6J mice as compared with mice receiving a control antibody and suppressed 6-h refeeding after 24 h of food deprivation. Indirect respiratory measures of metabolism after refeeding and relative fuel substrate utilization were unaffected. The results support the hypothesis that acylated ghrelin stimulates appetite and curbs energy expenditure during deficient energy intake, whereas *des*-acyl ghrelin does not potently share these functions. Catalytic anti-ghrelin antibodies might thereby adjunctively aid consolidation of caloric restriction-induced weight loss and might also be therapeutically relevant to Prader–Willi syndrome, characterized after infancy by hyperghrelinemia, hyperphagia, and obesity.

hormone inactivation | obesity | neuropeptides

Approximately 1 billion people worldwide are overweight or obese (body mass index = 25–29.9 or >30 kg/m^2, respectively) (1), conditions associated with significant morbidity and mortality and for which new treatments are needed (2). Among recently characterized molecules implicated in energy homeostasis, ghrelin was identified in 1999 during a search for the endogenous ligand for the GH secretagogue receptor (GHSR) (ghrelin receptor), previously localized to peripheral tissues and to several CNS sites, including the arcuate nucleus and ventromedial hypothalamic nucleus (3). Human ghrelin is a 28-amino acid acylated peptide (GSS(*n*-octanoyl)FLSPEHQRVQQRKESKKPPAKLQPR) released mainly from endocrine cells of the stomach and upper gastrointestinal tract but also expressed in testes, kidney, pituitary, pancreas, lymphocytes, and brain (3–7). Several findings establish gastric ghrelin as an indicator of energy insufficiency and anabolic modulator of energy homeostasis (3). Ghrelin secretion is thought to stimulate food intake, slow metabolic rate, and spare lipid oxidation via central (8) and, possibly, afferent vagus nerve-mediated modes of action (9). Human studies have found a preprandial rise and postprandial decline in plasma ghrelin levels, consistent with a role for ghrelin in hunger and meal initiation (7, 10). Indeed, circulating ghrelin levels are increased by food deprivation and decreased by meals, glucose load, insulin, and somatostatin (11–13). Pharmacological increases in ghrelin trigger food intake in rats or humans (6, 14–18) and decrease energy expenditure and the relative utilization of fat as an energy substrate, leading to weight gain and adiposity with chronic central administration (6, 13, 19, 20).

Although some early loss-of-function studies cast doubt on the physiological importance of ghrelin (21), recent studies suggest mice deficient for ghrelin or its receptor store less of their consumed food and are resistant to diet-induced obesity (22, 23). Ghrelin knockout mice expend more energy and locomote more (22), and GHSR1a receptor deficient mice show increased whole-body fat oxidation (23). Also, small-molecule GHSR1a antagonists reduce food intake, promote loss of weight selectively in the form of body fat, and acutely improve glucose tolerance by augmenting glucose-induced insulin secretion (24–30). Subchronic neutralization of the ghrelin molecule with the biostable aptamer l-NOX-B11-2, an l-RNA-spiegelmer based therapeutic, similarly reduced spontaneous food intake and feed efficiency, promoted weight loss, and reduced fat mass (31). Finally, active immunization with *N*-terminal-inclusive ghrelin haptens slowed weight gain and fat accrual in adult rats in direct relation to the elicited anti-ghrelin antibody response (32).

Ghrelin levels increase during voluntary energy restriction (i.e., dieting); these increases predict weight regain and are thought to impede sustained weight loss (7, 33–38), so new approaches to oppose ghrelin action may be therapeutically important. From a chemical standpoint, ghrelin is the first peptide isolated from natural sources in which the hydroxyl group of a Ser residue (Ser-3) is acylated by *n*-octanoic acid (3, 39). Octanoylation had not been observed in posttranslational peptide modification, but it is essential for the GH-releasing and potent GHSR1 agonist activity of ghrelin (3, 39, 40). Hence, degradation of the serine octanoate ester moiety of ghrelin provides an enticing target for obesity research.

Intracerebroventricular administration of polyclonal antibodies that bind ghrelin can suppress food intake (3, 8, 39). Of greater impact would be a catalytic antibody that could both sequester and/or degrade the ester moiety of ghrelin, and thus essentially

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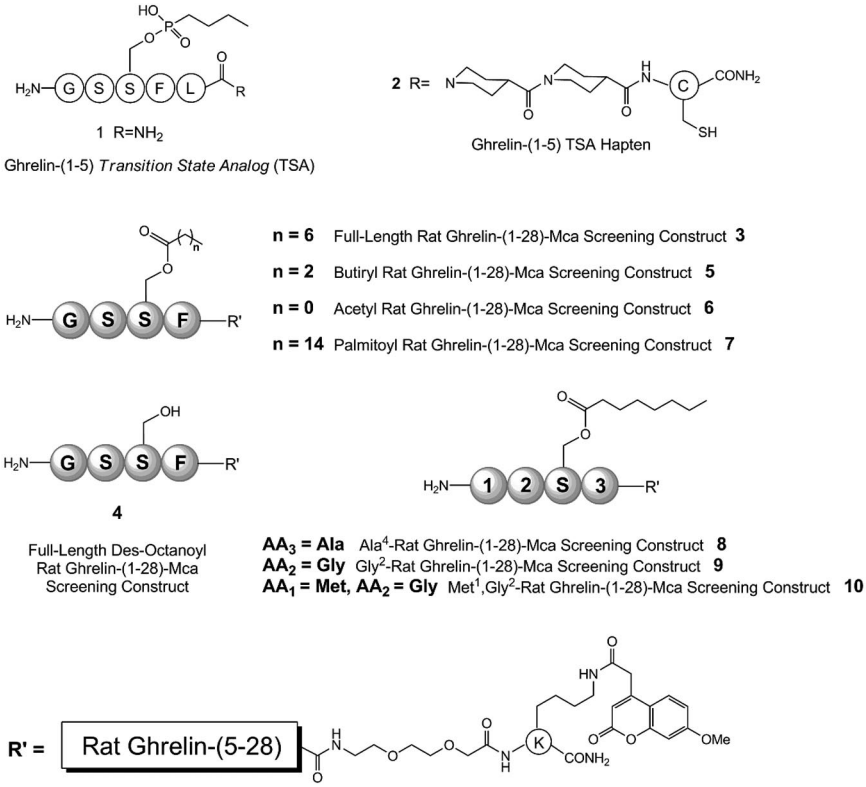

Fig. 1. Structures of ghrelin-derived peptides **1–10** synthesized for the generation, specificity and kinetic characterization of catalytic mAbs.

deactivate ghrelin. Herein, we describe the foundation for an anti-ghrelin immunopharmacotherapy based on catalytic antibodies that hydrolyze the Ser-3 octanoate ester moiety of ghrelin.

Results and Discussion

Monoclonal antibodies were obtained through immunization of mice with ghrelin phosphonate transition state analog **1**, conjugated to the immunogenic carrier protein keyhole limpet hemocyanin (KLH), through a covalent link between the thiol moiety of **1** and an *N*-maleimidomethyl cyclohexane-1-carboxylate cross-linker, resulting in hapten **2** (Fig. 1). The design of this hapten is based on methodology that is well-established in the field of catalytic antibodies (41, 42), in which phosphonate monoester **2** resembles the transition state of the hydrolysis reaction of the serine ester of ghrelin to its inactive *des*-octanoyl form. As a core antigen structure, we selected the first 5 *N*-terminal amino acids, partly based on studies that have evaluated truncated analogs of ghrelin, in which Ser^3(octanoyl)-ghrelin (1–5) was shown to be the shortest structural analog to display activity similar to the native hormone (39). Another consideration was that the antibody combining site can host up to 8 amino acid residues; as such, the octanoate ester as found in natural ghrelin was truncated to a smaller 4-carbon chain appendage. We have used this approach successfully in the past for antibodies generated against lipid A (43), because it aids in the minimization of lipid formation and increases antibody recognition of the entire haptenic structure. In addition, we extended the hapten with 2 isonipecotic acid (Isn) moieties as a rigid linker to generate a more focused immune response, and a cysteine residue was included to enable a high-yield conjugation to KLH (see above). Hapten **2** was synthesized on solid phase [supporting information (SI) Fig. S1, experimental details are provided in *Materials and Methods* and *SI Materials and Methods*] and was coupled to KLH through thioether conjugation chemistry; immunization of BALB/c mice with the immunoconjugate resulted in a panel of 19 monoclonal catalytic antibodies (mAbs) for analysis. All mAbs were purified from ascites, using ion-exchange and protein G affinity chromatography.

Our initial screen for catalysis for the hydrolysis of rat ghrelin to its *des*-octanoyl form by the panel of mAbs, with use of synthetic native rat ghrelin (experimental details are provided in *SI Materials and Methods*) indicated that several antibodies could accelerate the hydrolysis of native rat ghrelin. From this initial screen 3 mAbs demonstrated turn-over and were evaluated in greater detail. To perform expedient, safe and reliable kinetic analysis of the selected antibodies a new substrate **3** and *des*-octanoyl product **4** were designed (Fig. 1), based on the following criteria: (*i*) ghrelin's extinction coefficient is quite poor and impedes HPLC analysis at low concentration and (*ii*) the only alternative currently available that grants sensitive detection is ^{125}I-labeled ghrelin. Thus, **3** and **4** were prepared so as not to compromise the "wild-type" structure of ghrelin to increase sensitivity.

Using compounds **3** and **4** in this HPLC assay allowed us to identify the most proficient of the initially selected antibodies, of which mAb GHR-11E11 was assessed in detail (see Fig. 2 and Fig. S2). Well-behaved Michaelis–Menten kinetics were observed with this antibody (K_M = 2.4 μM, $k_{cat} = 2.59 \times 10^{-3}$ min^{-1}, k_{cat}/k_{uncat} = 120), which was competitively inhibited by transition state analog **1** (K_i = 0.14 μM—this value was calculated from the observed IC_{50} value, using a fixed substrate concentration and varying inhibitor concentration) (44). The thermodynamic K_i was determined from $K_{i,app}$ via the relationship for competitive kinetics: $K_i = K_{i,app}/(1 + [S]/K_M)$, where $[S]$ = 400 μM and K_M = 2.4 μM. We fully realize that the rate acceleration observed was not overly impressive; however, we note that this is the first example to our knowledge of antibody-catalyzed hydrolysis of an aliphatic long-chain alkyl ester. Interestingly, although turnover numbers were modest it appears more sophisticated chemistry in the antibody-combining site likely comes into play because the $K_M/K_i \approx k_{cat}/k_{uncat}$ relationship, according to a standard thermodynamic cycle based on TS theory (45), would predict a rate enhancement for mAb GHR-11E11 of

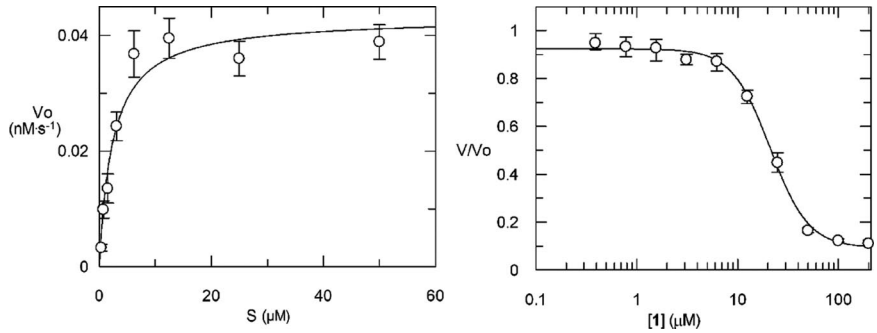

Fig. 2. Kinetic evaluation of catalyzed ghrelin hydrolysis. (*Left*) Catalysis of ghrelin hydrolysis by addition of 1 μM GHR-11E11 to varying concentrations of substrate **3** (0.4–50 μM) at 37 °C. (*Right*) Dose-response plot of inhibiton of ghrelin hydrolysis by 1 μM GHR-11E11 with varying inhibitor **1** concentrations; IC_{50}: 21.3μM.

only ≈15, 8-fold less than that actually observed (see Fig. 2 and Fig. S2).

The specificity of the mAb GHR-11E11 was examined using 6 synthetic ghrelin screening substrates **5**–**10**, which included Ser^3(butyryl)-, Ser^3(acetyl)-, and Ser^3(palmitoyl)-ghrelin-Mca peptides, and 3 Ser^3(octanoyl)-ghrelin-derived peptides with $Phe^4 \rightarrow Ala^4$, $Ser^2 \rightarrow Gly^2$, and Gly^1-$Ser^2 \rightarrow Met^1$-Gly^2 mutations in the *N*-terminal amino acid sequence (Fig. 1). The ghrelin peptides with variable length of the fatty acid ester chains were designed to determine the diapason of mAb GHR-11E11 catalytic ability toward hydrolysis of ghrelin ester peptides, whereas the ghrelin peptide analogs were used to establish the specificity of the catalytic mAb for native ghrelin in the presence of other endogenous *O*-acylated proteins and peptides. In summary, mAb GHR-11E11 was found to accelerate hydrolysis of the Ser^3(butyryl)-ghrelin construct **5**, and Michaelis–Menten kinetics were observed (K_M = 8.49 μM, $k_{cat} = 4.57 \times 10^{-3}$ min^{-1}, $k_{cat}/K_M = 9$ $M^{-1}{\cdot}s^{-1}$). This result is in line with the GHR-11E11-catalyzed hydrolysis kinetics of the Ser^3(octanoyl)-ghrelin substrate **3**, because this antibody was secured with a butylphosphonate ester hapten. At the same time, GHR-11E11 did not substantially alter the hydrolysis rates of the Ser^3(acetyl)- and Ser^3(palmitoyl)-ghrelin peptide analogs **6** and **7**. Catalytic antibody GHR-11E11 catalyzes the hydrolysis of ghrelin peptide ester analogs with a narrow range of the *O*-acyl group length, which includes both Ser^3(butiryl)- and Ser^3(octanoyl)-ghrelins, but the catalytic activity of the antibody drops rather abruptly with a shorter *O*-acyl group, and diminishes more gradually with increasing length of the *O*-acyl group. Furthermore, GHR-11E11 showed little ability to significantly catalyze hydrolysis of the ghrelin peptide analogs **8**–**10**, which suggests that this antibody is highly specific for the amino acid sequence of native ghrelin, and is unlikely to affect other endogenous *O*-acylated proteins and peptides.

The observed catalytic efficiency of antibody GHR-11E11 ($k_{cat}/K_M = 18$ $M^{-1}{\cdot}s^{-1}$) is modest; however, because of ghrelin's short half-life in mammals and because circulating plasma ghrelin concentrations have been estimated to be subnanomolar, a high catalytic proficiency may not be necessary to be of potential *in vivo* functional relevance. To determine the catalytic activity of GHR-11E11 *in vivo*, adult male C57BL/6J mice were administered GHR-11E11 ($n = 4$) or a control Ab (an anti-nicotine Ab; NIC-1 9D9, $n = 5$) intravenously by tail vein. Blood was collected from the submandibular vein into chilled polypropylene tubes containing EDTA, PMSF, and HCl to reduce degradation/desoctanoylation of ghrelin (46). As expected, baseline plasma levels of acylated (119.6 ± 24.6 vs. 93.3 ± 15.6 pg/mL) and *des*-acyl ghrelin (743.1 ± 86.8 vs. 680.7 ± 81.0 pg/mL), measured by specific ELISAs (Bio-Vendor), did not differ between groups. However, 15 min after treatment, acylated ghrelin levels decreased by 90 ± 18% in mice treated with GHR-11E11 ($P < 0.05$ 0%), a reduction not seen in control mice (23.3 ± 25.1 vs. 135.1 ± 35.0 pg/mL, respectively, $P < 0.05$) or in levels of *des*-acyl ghrelin (768.4 ± 79.0 vs. 635.3 ± 130.3 pg/mL). As a result, the ratio of acylated/*des*-acyl ghrelin was significantly lower in mice, which received the catalytic anti-ghrelin antibody (0.025 ± 0.039 vs. 0.186 ± 0.052, $P < 0.05$). Acylated, but not *des*-acyl, ghrelin levels also tended to be lower 24-h after administration in GHR-11E11-treated mice than in controls (103.5 ± 9.6 vs. 133.3 ± 12.1 pg/mL, respectively, $P = 0.07$). Interestingly, *in vitro* studies demonstrated that the catalytic activity of GHR-11E11 was entirely abrogated in the presence of 5 μM serine esterase inhibitor PMSF, in agreement with similar literature precedents (47, 48), also indicating that the antibody-induced reduction in acylated ghrelin levels occurred *in vivo*, rather than after blood collection.

We sought to determine whether i.v. administration of GHR-11E11 altered metabolic rate or refeeding in fasting mice, in which ghrelin activity is increased. Adult male C57BL/6J mice were surgically implanted with i.v. jugular catheters and allowed to recover. Then, mice were acclimated to individual open-circuit indirect calorimetry chambers equipped with computer-monitored food and water access and with photobeams to detect locomotor activity (Comprehensive Lab Animal Monitoring System, Columbus, OH) for at least 72 h. Matched for body weight (25.2 ± 0.5 vs. 25.2 ± 0.6 g), mice were intravenously administered (50 mg/kg) either the catalytic ghrelin Ab (GHR-11E11, $n = 8$) or the anti-nicotine control Ab ($n = 9$) within the first hour of the light cycle. Mice were then subjected to a 24-h fast, during which changes in metabolic rate and locomotor activity were monitored for 12 h. Fig. 3 shows that fasted ghrelin Ab-treated mice expended more energy ("heat") across the entire light cycle than did fasted mice treated with the control Ab ($F[1,14] = 20.90$, $P < 0.001$). Increased energy expenditure was reflected in increased oxygen consumption (VO_2; $F[1,14] = 22.57$, $P < 0.001$) and carbon dioxide production (VCO_2; $F[1,14] = 11.98$, $P < 0.005$). Groups did not differ in their relative energy substrate utilization, with values of the respiratory exchange ratio (RER ≈ 0.75) indicating greater utilization of lipid than carbohydrate in both groups, as expected from a period of fasting during the light cycle. Ghrelin Ab-treated mice showed more motor activity than controls during the first 2 h after treatment, but not thereafter (Hour × Treatment: $F(5, 70) = 5.94$, $P < 0.03$), the latter finding suggesting that differences in energy expenditure were at least partly independent from increased motor activity.

When provided access to chow beginning from the second hour of the next light cycle, mice treated ≈24 h earlier with GHR-11E11, the catalytic ghrelin Ab, showed blunted 6-h cumulative food intake (Fig. 4) as compared with mice previously treated with the control nicotine Ab (Treatment × Hour: $F[5,60] = 6.30$, $P < 0.001$).

In the hour before they were refed ("Unfed" in Fig. 4, corresponding to the first hour of the light cycle), mice treated with GHR-11E11 showed greater energy expenditure, VO_2 and VCO_2 than control-Ab treated mice. With refeeding, however, this difference was eliminated; the metabolic rate of refed, control Ab-treated mice rapidly rose to that of ghrelin Ab-treated mice. Refed groups also did not differ in their relative energy substrate utilization, with values of the respiratory exchange ratio rising to levels (RER≈0.9–0.96) indicating greater carbohydrate than lipid utili-

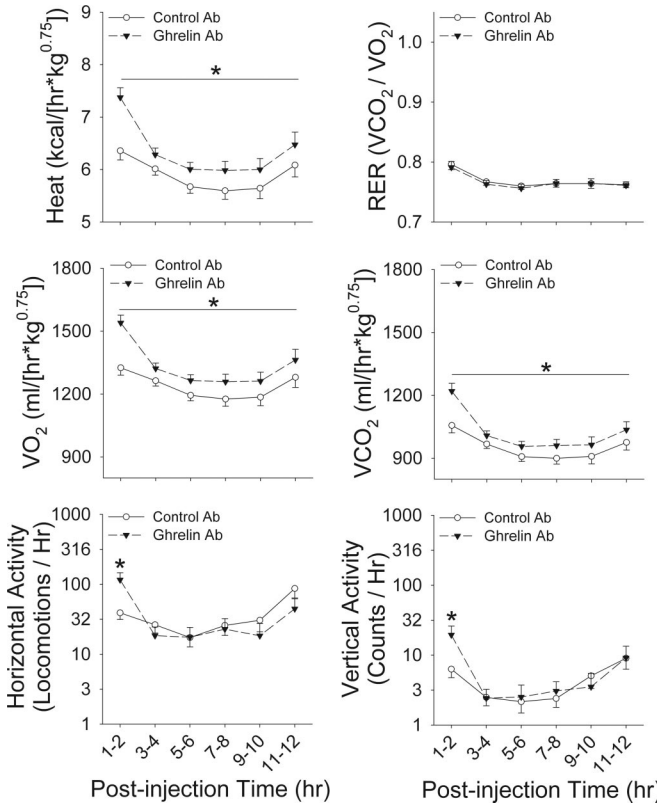

Fig. 3. Shown are the rate of energy expenditure (heat, *Top Left*), respiratory exchange ratio (RER) (*Top Right*), the rates of oxygen consumption (VO_2) (*Middle Left*) and carbon dioxide production (VCO_2) (*Middle Right*), and rates of horizontal and vertical motor activity (*Bottom*) in food-deprived, antibody-treated, adult male C57BL/6J mice tested in open-circuit indirect calorimetry chambers. Data are expressed in 2 h bins as $M \pm$ SEM across the 12-h light cycle. Mice received i.v. administration (i.v. 50 mg/kg) of a catalytic antibody against ghrelin ($n = 8$, GHR-11E11) or of an isotype-matched nicotine control Ab ($n = 9$, NIC-1 9D9) before data collection; *, $P < 0.05$ vs. control Ab-treated mice.

zation in both treatment groups, as expected from a period of refeeding (Fig. 5). Neither vertical nor horizontal motor activity of treated groups differed from one another (data not shown).

The immune system can be used to generate biocatalysts via its ability to produce antibodies to an infinite range of molecular

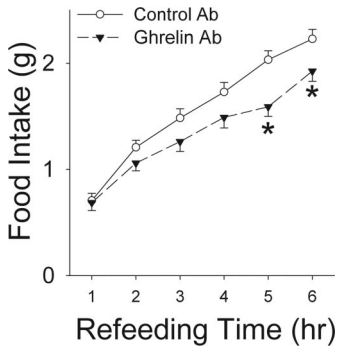

Fig. 4. Food intake in 24-h food-deprived adult male C57BL/6J mice that had received i.v. administration (i.v. 50 mg/kg) of a catalytic antibody against ghrelin ($n = 8$, GHR-11E11) or of an isotype-matched nicotine control Ab ($n = 9$, NIC-1 9D9) 24 h earlier. Data express $M \pm$ SEM cumulative food intake across 6 h of refeeding beginning from the light cycle onset. *, $P < 0.05$ vs. control Ab-treated mice.

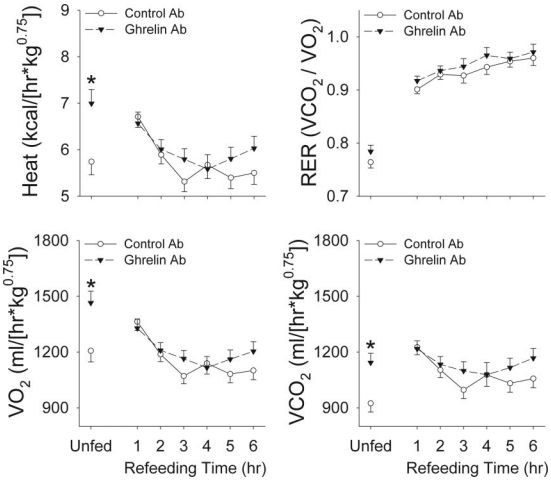

Fig. 5. Panels show the rate of energy expenditure (heat) (*Top Left*), respiratory exchange ratio (RER) (*Top Right*), rates of oxygen consumption (VO_2) (*Bottom Left*), and carbon dioxide production (VCO_2) (*Bottom Right*) in adult male C57BL/6J mice residing in open-circuit indirect calorimetry chambers. Data were collected in a 24-h food-deprived state ("Unfed") and during 6 h of refeeding on chow. Data are expressed in 1 h bins as $M \pm$ SEM. Mice had received i.v. administration (i.v. 50 mg/kg) of a catalytic antibody against ghrelin ($n = 8$, GHR-11E11) or of an isotype-matched nicotine control Ab ($n = 9$, NIC-1 9D9) 24 h before data collection; *, $P < 0.05$ vs. control Ab-treated mice.

structures, including those resembling transition states of chemical reactions. Simple binding antibodies typically only recognize antigen ground states with high affinity and specificity. However, by challenging the immune system with haptens mimicking the structure of the transition state of a given reaction, antibodies can be elicited that bind congruent substrates, thereby stabilizing the transition state and catalyzing the targeted reaction. Following this concept, antibodies have succeeded in the catalysis of >50 chemical transformations (49). Hydrolytic antibody catalysis represents a general platform from which therapeutics can potentially be developed. Previous targets for such catalytic antibodies included drugs of abuse, prodrug activation, and endogenous polypeptides (50).

Immunotherapeutic approaches against ghrelin may hold certain advantages over small-molecule GHSR1a antagonist treatments. First, immunoneutralization would occur outside of the central nervous system and therefore would not lead to potential centrally mediated side effects that may occur with blood–brain barrier-penetrating small molecule antagonists. Second, therapeutic antibodies use the bloodstream as a site of action, which affords a pharmacological profile of safety and efficacy that is highly predictable and reproducible. Third, immunization against a ligand may be more effective than small molecule receptor antagonist approaches when the receptor targets are unknown, multiple, or difficult to bind with specificity. This scenario is germane to ghrelin because some adipogenic effects of ghrelin have been proposed to be mediated by a non-GHSR1a receptor (51) and in which other ghrelin binding sites are known (GHSR1b) or suspected (52, 53). Fourth, dosing would need to be less frequent with an immunization approach because of the extended half-life of antibodies *in vivo*. Of course, given the complex and recalcitrant nature of obesity and associated comorbidities, any antibody-based strategy would likely best be used in combination with other available drugs and as part of a comprehensive treatment approach that includes nutritional, exercise, educational, and psychosocial components.

The present proof-of-concept data suggest that passive immunopharmacotherapy with a catalytic anti-ghrelin antibody such as GHR-11E11 can both decrease the serum ghrelin/desacyl ghrelin ratio and modulate energy homeostasis. Ghrelin, an

endogenous peptide ligand for the GHSR1a receptor released into circulation from the stomach, is posttranslationally acylated by the addition of octanoic acid to the Ser-3 residue. This modification is critical for ghrelin's active transport across the blood–brain barrier (54) and potent GHSR1a activity (55). Whereas infusion of acylated ghrelin acutely stimulates food intake and subjective hunger (17, 56) and chronic administration causes weight gain (6), administration of the hydrolytic degradation product *des*-acyl ghrelin either does not stimulate appetite or does so less effectively than acylated ghrelin (57). Here, administration of a catalytic antibody that can both bind and degrade ghrelin to its *des*-acyl form maintained a relatively increased metabolic rate in fasting mice suppressed refeeding after food deprivation. The results support the hypothesis that acylated ghrelin physiologically stimulates appetite and curbs whole-body energy expenditure during periods of deficient energy intake, whereas, contrary to some previous reports (57), *des*-acyl ghrelin does not potently share these functions.

One question raised by the present study is what might be required parameters for a functionally relevant catalytic antibody in humans. Ghrelin concentrations in human serum have been reported to be between 0.1–2 nM (7, 10, 49, 58, 59). Although it is difficult to estimate the needed degradation efficiency to attenuate ghrelin's "gut-brain axis" effects on energy homeostasis, a quantitative study on ghrelin dynamics across a day in dieting obese subjects provides starting insights (10). Based on this study, to an initial approximation, there are 3 components to ghrelin kinetics: a linear (zero order) formation of ghrelin that initiates upon voiding the stomach, a suppression of this rate when the stomach is distended, and an overall first-order elimination pathway for ghrelin. The net formation rate of ghrelin in humans is $\approx$20 pM·h^{-1}, and its elimination is 0.12 h^{-1} ($t^{1/2}$ = 6 h). On average, feeding occurs when the plasma ghrelin concentration reaches $\approx$180 pM. Upon feeding, ghrelin production ceases, and in a first-order process, the plasma concentration of ghrelin drops to an average of 120 pM before ghrelin production resumes.

Thus, preventing the plasma ghrelin concentration from rising >140 pM might attenuate feeding responses. To maintain a 140 pM plasma concentration, a catalytic antibody would need to consume ghrelin at 20 pM·h^{-1} at 140 pM ghrelin. Given a reasonable therapeutic plasma concentration of catalytic antibody of 1 μM (60) the catalytic efficiency (k_{cat}/K_M) necessary to maintain plasma ghrelin at 140 pM can be roughly approximated from Eq. **1** as $\approx$40 M^{-1}s^{-1}, slightly more efficient than the degradation parameters observed for GHR-11E11 *in vitro* (k_{cat}/K_M = 18 M^{-1}·s^{-1}).

$$[\text{antibody}]\,\frac{k_{cat}}{K_M}\,[\text{ghrelin}] = net\ rate_{\text{formation}} \qquad [1]$$

Several limitations of the present study bear mentioning. First, antibodies were administered to lean animals and the functional significance of degrading ghrelin in obese animals, in which circulating levels are reduced, are unclear. Relevant here, however, ghrelin levels increase with weight loss and predict subsequent weight regain. Second, the effects of the catalytic antibody were only studied acutely in fasted animals. Resulting issues of future interest include potential compensatory mechanisms, effects of ghrelin degradation in fed animals, long-term effects of chronically degrading ghrelin on non-energy homeostasis-related functions of ghrelin, and the potential of increasing non-GHSR1a-mediated actions of the hydrolysis product, *des*-acyl ghrelin (55, 61–66). Each of these caveats, except the last, is not unique to ghrelin degradation immunopharmacotherapy and applies also to small-molecule GHSR1a antagonists. Third, regarding potential ghrelin insufficiency, therapeutic dosing would aim to reduce, not eliminate, circulating acylated ghrelin. Much is known regarding life with chronically reduced ghrelin levels because thousands of individuals have undergone Roux-en-Y gastric bypass surgery, which reduces ghrelin levels by 50–70% for weeks relative to weight-loss controls. Postsurgical ghrelin levels are even lower after total gastrectomy or sleeve gastrectomy (removal of the fundus) and remain so in the long-term (34–38, 67–77). The Roux-en-Y gastric bypass and sleeve gastrectomy surgeries both produce massive weight reduction, greater than those of other surgical procedures that less effectively reduce ghrelin levels and improve the health of previously morbidly obese patients with low complication rates (37, 78, 79). Finally, the mechanisms, which led to reduced energy expenditure and refeeding were not investigated. Reduced recruitment of GHSR1a-expressing cells in the mediobasal hypothalamus and afferent vagus are suspected, but this remains to be demonstrated.

In summary, we have produced antibodies can facilitate hydrolysis of the serine octanoate ester moiety of ghrelin. Administration of an exemplar catalytic antibody modulated energy homeostasis *in vivo*, maintaining greater whole body energy expenditure during fasting and reducing subsequent refeeding in mice. As such, catalytic anti-ghrelin antibodies might adjunctively aid attainment and consolidation of caloric restriction-induced weight loss. Ghrelin degradation might also be therapeutically relevant to Prader–Willi syndrome, characterized after infancy by hyperghrelinemia, hyperphagia, and progressive obesity (10). Additional studies will further define the role of immunopharmacotherapy in perturbing energy homeostasis and ultimately in combating obesity.

Materials and Methods

Peptide Synthesis. The hapten and substrates were prepared on a 1.0-mmol scale as C-terminal amides, using Rink amide AM resin and conventional Fmoc/tBu SPPS protocols with standard DIC/6-Cl-HOBt coupling chemistry (*SI Materials and Methods*) on a CS Bio 136 automated peptide synthesizer (CS Bio). The Ser^3 side chain esterifications were accomplished via DIC/DMAP chemistry for n-butylphosphonation, with carboxylic acid anhydride/pyridine for acetylation and butyrylation, and via PyBrOP/DIPEA activation chemistry for octanoylation and palmitoylation. Purifications were achieved by RP-HPLC.

Antibody Catalysis. Selection of monoclonal catalytic antibodies (mAbs) was performed by HPLC detection of *des*-octanoyl ghrelin formation upon incubation of native rat ghrelin with each member of a panel of 19 mAbs (10 μM) at 37 °C in phosphate buffered saline (0.5 mL), pH 7.4, for 10 h, using the analytical HPLC method. Further characterization of selected catalytic antibodies was performed by incubation of the selected mAb (1 μM) with varying concentrations of methoxycoumarin-modified ghrelin analogs **3** and **5** (at 0.4, 0.8, 1.6, 3.1, 6.2, 12.5, 25, and 50 μM) for 5 h at 37 °C in phosphate buffered saline, pH 7.4, with subsequent HPLC analysis. Formation of product **4** was monitored by RP HPLC, with detection at 324 nm. Inhibition of catalysis by TS analog **2** was measured by HPLC after incubation of **3** (400 μM) and mAb GHR-11E11 (1 μM) in the presence of varying concentrations of **2** (0 to 200 μM) for 5 h. The difference between the extent of catalyzed and uncatalyzed hydrolysis of the ghrelin substrates provided the basis for determining of the specificity of GHR-11E11 (*SI Materials and Methods*).

Subjects. Mature male C57BL/6J mice (n = 17) (The Jackson Laboratory) were group-housed in a 12h:12h lit (0600h lights on), humidity- (60%) and temperature-controlled (22 °C) vivarium with continuous access to chow (LM-485 Diet 7012; Harlan Teklad) and water unless mentioned otherwise. After surgical implantation of jugular catheters under isoflurane anesthesia (1–3% in oxygen), mice were allowed to recover for at least 1 week and then acclimated to indirect calorimetry chambers (*SI Materials and Methods*). Procedures adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication number 85–23, revised 1996) and the ''Principles of laboratory animal care'' (www.nap.edu/readingroom/bookslabrats) and were approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute.

Metabolic and Food Intake Study. Indirect calorimetry was performed in acclimated (>72 h), singly housed, mice using a computer-controlled, open-circuit system (Oxymax System) that was part of an integrated Comprehensive Lab Animal Monitoring System (Columbus Instruments) (*SI Materials and Methods*).

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1. Yach D, Stuckler D, Brownell KD (2006) Epidemiologic and economic consequences of the global epidemics of obesity and diabetes. *Nat Med* 12:62–66.
2. Allison DB, Fontaine KR, Manson JE, Stevens J, VanItallie TB (1999) Annual deaths attributable to obesity in the United States. *Jama* 282:1530–1538.
3. Kojima M, *et al.* (1999) Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 402:656–660.
4. Mori K, *et al.* (2000) Kidney produces a novel acylated peptide, ghrelin. *FEBS Lett* 486:213–216.
5. Tena-Sempere M, *et al.* (2002) Novel expression and functional role of ghrelin in rat testis. *Endocrinology* 143:717–725.
6. Tschop M, Smiley DL, Heiman ML (2000) Ghrelin induces adiposity in rodents. *Nature* 407:908–913.
7. Cummings DE, *et al.* (2002) Elevated plasma ghrelin levels in Prader–Willi syndrome. *Nat Med* 8:643–644.
8. Nakazato M, *et al.* (2001) A role for ghrelin in the central regulation of feeding. *Nature* 409:194–198.
9. De Smet B, *et al.* (2006) Energy homeostasis and gastric emptying in ghrelin knockout mice. *J Pharmacol Exp Ther* 316:431–439.
10. Cummings DE, *et al.* (2002) Plasma ghrelin levels after diet-induced weight loss or gastric bypass surgery. *N Engl J Med* 346:1623–1630.
11. Broglio F, *et al.* (2003) Ghrelin and the endocrine pancreas. *Endocrine* 22:19–24.
12. Deghenghi R, Broglio F, Papotti M, Muccioli G, Ghigo E (2003) Targeting the ghrelin receptor: Orally active GHS and cortistatin analogs. *Endocrine* 22:13–18.
13. Muccioli G, *et al.* (2002) Neuroendocrine and peripheral activities of ghrelin: Implications in metabolism and obesity. *Eur J Pharmacol* 440:235–254.
14. Espelund U, Hansen TK, Orskov H, Frystyk J (2003) Assessment of ghrelin. *APMIS Suppl* 140–145.
15. Kamegai J, *et al.* (2001) Chronic central infusion of ghrelin increases hypothalamic neuropeptide Y and Agouti-related protein mRNA levels and body weight in rats. *Diabetes* 50:2438–2443.
16. Shintani M, *et al.* (2001) Ghrelin, an endogenous growth hormone secretagogue, is a novel orexigenic peptide that antagonizes leptin action through the activation of hypothalamic neuropeptide Y/Y1 receptor pathway. *Diabetes* 50:227–232.
17. Wren AM, *et al.* (2001) Ghrelin enhances appetite and increases food intake in humans. *J Clin Endocrinol Metab* 86:5992.
18. Wren AM, *et al.* (2001) Ghrelin causes hyperphagia and obesity in rats. *Diabetes* 50:2540–2547.
19. Woods SC (2004) Gastrointestinal satiety signals I. An overview of gastrointestinal signals that influence food intake. *Am J Physiol Gastrointest Liver Physiol* 286:G7–13.
20. Yasuda T, Masaki T, Kakuma T, Yoshimatsu H (2003) Centrally administered ghrelin suppresses sympathetic nerve activity in brown adipose tissue of rats. *Neurosci Lett* 349:75–78.
21. Sun Y, Ahmed S, Smith RG (2003) Deletion of ghrelin impairs neither growth nor appetite. *Mol Cell Biol* 23:7973–7981.
22. Wortley KE, *et al.* (2005) Absence of ghrelin protects against early-onset obesity. *J Clin Invest* 115:3573–3578.
23. Zigman JM, *et al.* (2005) Mice lacking ghrelin receptors resist the development of diet-induced obesity. *J Clin Invest* 115:3564–3572.
24. Demange L, *et al.* (2007) Synthesis and pharmacological in vitro and in vivo evaluations of novel triazole derivatives as ligands of the ghrelin receptor. 1. *J Med Chem* 50:1939–1957.
25. Dezaki K, *et al.* (2006) Blockade of pancreatic islet-derived ghrelin enhances insulin secretion to prevent high-fat diet-induced glucose intolerance. *Diabetes* 55:3486–3493.
26. Esler WP, *et al.* (2007) Small-molecule ghrelin receptor antagonists improve glucose tolerance, suppress appetite, and promote weight loss. *Endocrinology* 148:5175–5185.
27. Helmling S, Jarosch F, Klussmann S (2006) The promise of ghrelin antagonism in obesity treatment. *Drug News Perspect* 19:13–20.
28. Moulin A, Ryan J, Martinez J, Fehrentz JA (2007) Recent Developments in Ghrelin Receptor Ligands. *ChemMedChem* 2:1242–1259.
29. Rudolph J, *et al.* (2007) Quinazolinone Derivatives as Orally Available Ghrelin Receptor Antagonists for the Treatment of Diabetes and Obesity. *J Med Chem* 50:5202–5216.
30. Serby MD, *et al.* (2006) 2,4-diaminopyrimidine derivatives as potent growth hormone secretagogue receptor antagonists. *J Med Chem* 49:2568–2578.
31. Shearman LP, *et al.* (2006) Ghrelin neutralization by a ribonucleic acid-SPM ameliorates obesity in diet-induced obese mice. *Endocrinology* 147:1517–1526.
32. Zorrilla EP, *et al.* (2006) Vaccination against weight gain. *Proc Natl Acad Sci USA* 103:13226–13231.
33. Kojima M, Kangawa K (2005) Ghrelin: Structure and function. *Physiol Rev* 85:495–522.
34. Korner J, *et al.* (2005) Effects of Roux-en-Y gastric bypass surgery on fasting and postprandial concentrations of plasma ghrelin, peptide YY, and insulin. *J Clin Endocrinol Metab* 90:359–365.
35. Korner J, *et al.* (2006) Differential effects of gastric bypass and banding on circulating gut hormone and leptin levels. *Obesity (Silver Spring, MD)* 14:1553–1561.
36. Langer FB, *et al.* (2005) Sleeve gastrectomy and gastric banding: Effects on plasma ghrelin levels. *Obes Surg* 15:1024–1029.
37. Shah M, Simha V, Garg A (2006) Review: Long-term impact of bariatric surgery on body weight, comorbidities, and nutritional status. *J Clin Endocrinol Metab* 91:4223–4231.
38. Stylopoulos N, Davis P, Pettit JD, Rattner DW, Kaplan LM (2005) Changes in serum ghrelin predict weight loss after Roux-en-Y gastric bypass in rats. *Surg Endosc* 19:942–946.
39. Bednarek MA, *et al.* (2000) Structure-function studies on the new growth hormone-releasing peptide, ghrelin: Minimal sequence of ghrelin necessary for activation of growth hormone secretagogue receptor 1a. *J Med Chem* 43:4370–4376.
40. Gauna C, *et al.* (2007) Unacylated ghrelin acts as a potent insulin secretagogue in glucose-stimulated conditions. *Am J Physiol Endocrinol Metab* 293:E697–E704.
41. Lerner RA, Barbas CF, III, Janda KD (1996) Making enzymes. *Harvey lectures* 92:1–40.
42. Schultz PG, Lerner RA (2002) Antibody catalysis: Completing the circle. *Nature* 418:485.
43. Jones LH, *et al.* (2002) Active immunization with a glycolipid transition state analogue protects against endotoxic shock. *Angew Chem Int Ed Engl* 41:4241–4244.
44. Hasserodt J, Janda KD, Lerner RA (2000) A class of 4-aza-lithocholic acid-derived haptens for the generation of catalytic antibodies with steroid synthase capabilities. *Bioorg Med Chem* 8:995–1003.
45. Wirsching P, Ashley JA, Lo CH, Janda KD, Lerner RA (1995) Reactive immunization. *Science* 270:1775–1782.
46. De Vriese C, Hacquebard M, Gregoire F, Carpentier Y, Delporte C (2007) Ghrelin interacts with human plasma lipoproteins. *Endocrinology* 148:2355–2362.
47. Ponomarenko NA, *et al.* (2007) Anti-idiotypic antibody mimics proteolytic function of parent antigen. *Biochemistry* 46:14598–14609.
48. Johnson G, Moore SW (2000) Cholinesterase-like catalytic antibodies: Reaction with substrates and inhibitors. *Mol Immunol* 37:707–719.
49. Ng PC, *et al.* (2004) Plasma ghrelin and resistin concentrations are suppressed in infants of insulin-dependent diabetic mothers. *J Clin Endocrinol Metab* 89:5563–5568.
50. Amir R, Shabat D (2005) Medicinal potential of catalytic antibodies. *Catalytic Antibodies*, ed Keinan E (Wiley-VCH, Weinheim, Germany).
51. Thompson NM, *et al.* (2004) Ghrelin and des-octanoyl ghrelin promote adipogenesis directly in vivo by a mechanism independent of the type 1a growth hormone secretagogue receptor. *Endocrinology* 145:234–242.
52. Beaumont NJ, *et al.* (2003) Ghrelin can bind to a species of high density lipoprotein associated with paraoxonase. *J Biol Chem* 278:8877–8880.
53. Muccioli G, Baragli A, Granata R, Papotti M, Ghigo E (2007) Heterogeneity of ghrelin/growth hormone secretagogue receptors. Toward the understanding of the molecular identity of novel ghrelin/GHS receptors. *Neuroendocrinology* 86:157–164.
54. Banks WA, Tschop M, Robinson SM, Heiman ML (2002) Extent and direction of ghrelin transport across the blood-brain barrier is determined by its unique primary structure. *J Pharmacol Exp Ther* 302:822–827.
55. Gauna C, *et al.* (2007) Unacylated ghrelin is not a functional antagonist but a full agonist of the type 1a growth hormone secretagogue receptor (GHS-R). *Mol Cell Endocrinol* 274:30–34.
56. Druce MR, *et al.* (2005) Ghrelin increases food intake in obese as well as lean subjects. *Int J Obes (London)* 29:1130–1136.
57. Toshinai K, *et al.* (2006) Des-acyl ghrelin induces food intake by a mechanism independent of the growth hormone secretagogue receptor. *Endocrinology* 147:2306–2314.
58. De Vriese C, *et al.* (2004) Ghrelin degradation by serum and tissue homogenates: Identification of the cleavage sites. *Endocrinology* 145:4997–5005.
59. Farquhar J, *et al.* (2003) Elevated umbilical cord ghrelin concentrations in small for gestational age neonates. *J Clin Endocrinol Metab* 88:4324–4327.
60. Chen CY, *et al.* (2005) Intracisternal des-acyl ghrelin inhibits food intake and non-nutrient gastric emptying in conscious rats. *Int J Mol Med* 16:695–699.
61. Ariyasu H, *et al.* (2005) Transgenic mice overexpressing des-acyl ghrelin show small phenotype. *Endocrinology* 146:355–364.
62. Baldanzi G, *et al.* (2002) Ghrelin and des-acyl ghrelin inhibit cell death in cardiomyocytes and endothelial cells through ERK1/2 and PI 3-kinase/AKT. *J Cell Biol* 159:1029–1037.
63. Bedendi I, *et al.* (2003) Cardiac effects of ghrelin and its endogenous derivatives des-octanoyl ghrelin and des-Gln14-ghrelin. *Eur J Pharmacol* 476:87–95.
64. Gauna C, *et al.* (2005) Ghrelin stimulates, whereas des-octanoyl ghrelin inhibits, glucose output by primary hepatocytes. *J Clin Endocrinol Metab* 90:1055–1060.
65. Muccioli G, *et al.* (2004) Ghrelin and des-acyl ghrelin both inhibit isoproterenol-induced lipolysis in rat adipocytes via a non-type 1a growth hormone secretagogue receptor. *Eur J Pharmacol* 498:27–35.
66. Tsubota Y, Owada-Makabe K, Yukawa K, Maeda M (2005) Hypotensive effect of des-acyl ghrelin at nucleus tractus solitarii of rat. *Neuroreport* 16:163–166.
67. Fruhbeck G, Diez Caballero A, Gil MJ (2004) Fundus functionality and ghrelin concentrations after bariatric surgery. *N Engl J Med* 350:308–309.
68. Fruhbeck G, *et al.* (2004) The decrease in plasma ghrelin concentrations following bariatric surgery depends on the functional integrity of the fundus. *Obes Surg* 14:606–612.
69. Fruhbeck G, *et al.* (2004) Fasting plasma ghrelin concentrations 6 months after gastric bypass are not determined by weight loss or changes in insulinemia. *Obes Surg* 14:1208–1215.
70. Geloneze B, *et al.* (2003) Ghrelin: A gut-brain hormone: Effect of gastric bypass surgery. *Obes Surg* 13:17–22.
71. Leonetti F, *et al.* (2003) Different plasma ghrelin levels after laparoscopic gastric bypass and adjustable gastric banding in morbid obese subjects. *J Clin Endocrinol Metab* 88:4227–4231.
72. Lin E, *et al.* (2004) The effects of gastric surgery on systemic ghrelin levels in the morbidly obese. *Arch Surg* 139:780–784.
73. Morinigo R, *et al.* (2004) Short-term effects of gastric bypass surgery on circulating ghrelin levels. *Obes Res* 12:1108–1116.
74. Stoeckli R, Chanda R, Langer I, Keller U (2004) Changes of body weight and plasma ghrelin levels after gastric banding and gastric bypass. *Obes Res* 12:346–350.
75. Sundbom M, Holdstock C, Engstrom BE, Karlsson FA (2007) Early changes in ghrelin following Roux-en-Y gastric bypass: Influence of vagal nerve functionality? *Obes Surg* 17:304–310.
76. Takachi K, *et al.* (2006) Postoperative ghrelin levels and delayed recovery from body weight loss after distal or total gastrectomy. *J Surg Res* 130:1–7.
77. Tritos NA, *et al.* (2003) Serum ghrelin levels in response to glucose load in obese subjects post-gastric bypass surgery. *Obes Res* 11:919–924.
78. Baxter JN (2007) Obesity: Surgical management. *Br J Hosp Med (London)* 68:85–88.
79. Kendrick ML, Dakin GF (2006) Surgical approaches to obesity. *Mayo Clin Proc* 81:S18–S24.